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Attorney Docket No. 18217-527



# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

**This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c)**

## INVENTORS / APPLICANTS

| Given Name (last name, first name, and middle initial [if any]) | | | Residence (City and State or Foreign Country) |
|---|---|---|---|
| Miranda | Maria | G. | Waco, TX 76798 |
| Pinney | Kevin | G. | Waco, TX 76798 |
| Dorsey | James | M. | Waco, TX 76798 |

## TITLE OF THE INVENTION

**NOVEL SEROTONIN REUPTAKE INHIBITORS**

## CORRESPONDENCE ADDRESS

| | |
|---|---|
| Attorney Name:<br>Firm Name and Address: | Ivor R. Elrifi, Ph.D.<br>MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO, P.C.<br>One Financial Center<br>Boston, MA 02111 |
| Telephone: | (617) 542-6000 |
| Fax: | (617) 542-2241 |

## ENCLOSED APPLICATION PARTS

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- ☐ **Sequence Listing** — Number of Pages:
- ☐ **Drawings** ( ☐ Formal; ☐ Informal) — Number of Sheets:
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- ☒ No.
- ☐ Yes, the name of the U.S. Government agency and the Government contract number are:

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Respectfully submitted,

Date: March 26, 2004

Ivor R. Elrifi, Registration No. 39,529
MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO, P.C.
One Financial Center
Boston, MA 02111
Telephone: (617) 542-6000

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# Synthesis of Novel Selective Serotonin Reuptake Inhibitors (SSRIs): Targeting SERT and 5-HT$_{2A}$ Receptors

***M. Graciela Miranda, Michael S. Stewart, and Kevin G. Pinney***

*Department of Chemistry and Biochemistry, and The Center for Drug Discovery, Baylor University, Waco, TX 76798-7348, USA*

**Abstract.** Since the 1980's, clinical use of selective serotonin reuptake inhibitors (SSRIs) in the treatment of depression has revealed that SSRIs are also effective against the symptoms of anxiety coexistent with depression. Evidence indicates the serotonin type 2A receptors play an important role in anxiety, depression, anorexia nervosa, and other disorders. Moreover, researchers have found that blockade of the 5-HT2A receptor may restore some of the undesired side effects of SSRIs. The proposed research focuses on the development of novel bi-functional molecules that, by exhibiting an enhanced antagonism towards the 5-HT2A receptors while keeping a highly selective inhibition of serotonin reuptake activity, provide synergism in their potential efficacy over a wider variety of both depressive and anxiety disorders. In order to achieve our goal, we have designed and plan to prepare a series of molecules which judiciously combine portions of known 5-HT2A receptor antagonists with portions of SSRI's such as fluoxetine.

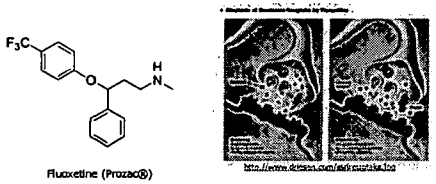
## Introduction

**About Depression.-** Depression is a common, life-disrupting, potentially lethal illness that can affect both sexes and all ages. Untreated major depression remains a serious public health problem ands its incidences are staggering. Its peak onset is in the early adult years. Suicide occurs in as many as 15% of patients with depression, especially those with recurrent episodes and hospitalizations. Therefore it becomes evident that treatment of depression is a matter of prime importance.[1]

Depression has no single cause; often, it results from a combination of factors. Whatever its cause, depression is not just a state of mind. It is related to physical changes in the brain, and connected to an imbalance of a type of chemical that carries signals in the brain and nerves. These chemicals are called neurotransmitters. Among the most important neurotransmitters related with depression are serotonin (5-HT), norepinephrine (NE) and dopamine (DA). Serotonin plays a very important role in the mood disorders, specially in anxiety and depression, aggression and impulsivity. Regulation of the mood disorders is possible either by agonistic or antagonistic action on a certain type of the serotonin receptors.

**Treatment Options.-** Selective serotonin reuptake inhibitors (SSRIs) have demonstrated efficacy in the treatment of depression and anxiety disorders, and they are currently the treatment of choice.[2] SSRIs are well tolerated, easier to manage, and have fewer drug interactions than older antidepressants. Evidence indicates that the serotonin (5-HT) neurons in the limbic system (i.e. the mood center of the brain) of depressed patients fail to release an adequate amount of 5-HT to sustain normal postsynaptic receptor activation. Inhibition of the reuptake of 5-HT is believed to compensate for this diminished neurotransmitter release.[1]

Consistent with this idea, SSRIs exert their therapeutic effect by blocking the reuptake of serotonin into the presynaptic nerve terminal, thus increasing the synaptic concentration of serotonin.[2] Fluoxetine (Prozac®) is one of the most representative molecules of this kind and it's a highly selective serotonin reuptake inhibitor.[3]



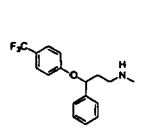
Fluoxetine (Prozac®)

It is also believed that SSRIs increase the efficacy of the serotonin (5-HT) neurons by desensitizing 5-HT autoreceptors located on the presynaptic 5-HT nerve terminals. The ability of the 5-HT autoreceptors to inhibit the release of 5-HT decreases after long-term treatment with SSRIs, with the net effect being that a greater amount of 5-HT is released per impulse. The release of 5-HT from these neurons, thus, becomes more efficient.[4]

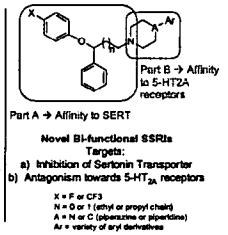
**The Complex Serotonin System.-** However, the serotonin system in the brain is very complex. During the last decade multiple serotonin receptor subtypes have been characterized. Evidence indicates that the serotonin type 2A receptors (5-HT$_{2A}$) play an important role in anxiety, depression, anorexia nervosa, migraine, hypertension and other cardiovascular disorders.[5] Moreover, researchers have found that blockade of the 5-HT$_{2A}$ receptor may restore some of the undesired side effects of SSRI's, such as sexual dysfunction.[6] Fluoxetine binds to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, although there is controversy as to whether fluoxetine acts as an agonist or antagonist at those receptors. Antagonism at 5-HT$_{2A}$ receptors, however, may explain the appetite-suppressant affects of fluoxetine.[2]

Based on these observations, it is our hypothesis that by merging into one molecular entity an enhanced 5-HT$_{2A}$ receptor antagonism while maintaining a highly selective serotonin reuptake inhibition, a superior pharmacological treatment for depression and anxiety disorders may be achieved.

## Rationale for Drug Design



Fluoxetine (Prozac®)
Target: Inhibition of Sertonin Transporter

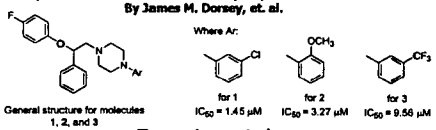



## Objectives

- To design and synthesize a library of twenty or more novel bi-functional organic molecules which combine 5-HT2A receptor antagonism and serotonin transporter reuptake inhibition into one molecular entity
- To provide biological evaluation of the synthesized molecules with respect to the affinity of the above molecules to their respective targets and a comparison with currently used psychotherapeutics
- To build a data base that will lead to the development of clinically relevant modern psychotherapeutic medications, which not only ameliorate the symptoms of depression but also enhance the positive response of individuals to other forms of intervention, such as psychotherapy.

## Previous Studies

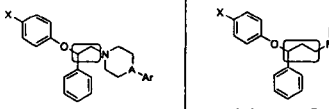
**Synthesis and Biological Evaluation of 2-(4-Fluorophenoxy)-2-phenyl-ethyl Piperazines as Serotonin-Selective Reuptake Inhibitors with a Potentially Improved Adverse Reaction Profile**
**By James M. Dorsey, et. al.**




## Experimental

Our chemical strategy for constructing this hybrid (bi-functional) molecules from 5-HT$_{2A}$ antagonists and serotonin reuptake inhibitors will be to covalently couple functionalized piperazines and piperidines with Fluoxetine and Fluoxetine homologues.

- **Homologues Type I**



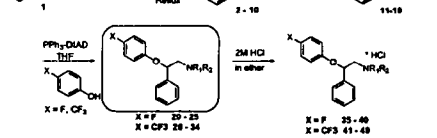
**Aminoethane Derivatives**
X = F or CF3
A = N or C (piperazine or piperidine)
Ar = variety of aryl groups

- **Homologues Type II**

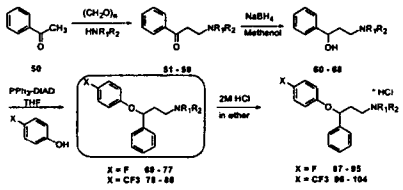


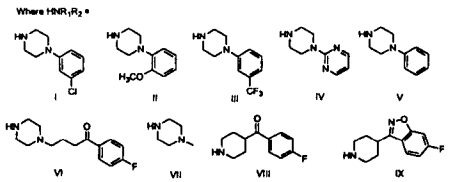
**Aminopropane Derivatives**
X = F or CF3
A = N or C (piperazine or piperidine)
Ar = variety of aryl groups

**Scheme 1.** Synthesis of Fluoxetine Homologues Type I – 2-[(4-aryloxy)-2-phenyl]-1-aminoethane Derivatives

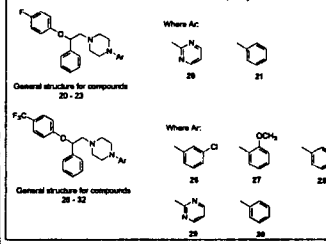



**Scheme 2.** Synthesis of Fluoxetine Homologues Type II – 3-[(4-aryloxy)-3-phenyl]-1-aminopropane Derivatives

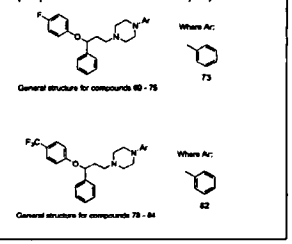



Homologues Type I - 2-[(4-aryloxy)-2-phenyl]-1-aminoethane Derivatives Already Synthesized




Homologues Type I - 3-[(4-aryloxy)-3-phenyl]-1-aminopropane Derivatives Already Synthesized




## Biological Evaluation

- The hydrochloride (HCl) salts of compounds 21, 26, 27, 28, 30, 73 and 82 have been sent for biological evaluation to the National Institutes of Health Psychoactive Drug Screening Program (NIH-PDSP) at Case Western Reserve University in Cleveland, OH.
- Their affinity towards the SERT will be evaluated, as well as their affinity and agonistic/antagonistic activity towards the 5-HT2A receptors. For screening purposes, their activity towards the serotonin 5-HT1A receptors will also be evaluated.
- Biological evaluation will provide valuable information for Structure-Activity Relationship studies, as well as aid in future potential target design.

## Conclusions

The proposed research focuses on the development of novel bi-functional molecules that, by exhibiting an enhanced antagonism towards the 5-HT$_{2A}$ receptors while keeping a highly selective inhibition of serotonin reuptake, provide synergism in terms of their potential efficacy over a wider variety of both depressive and anxiety disorders. In order to develop and evaluate molecules which can concomitantly antagonize the 5-HT$_{2A}$ receptors and block the serotonin transporter system, we have designed and prepared a new series of molecules which judiciously combine portions of known 5-HT$_{2A}$ receptor antagonists with portions of SSRI's such as fluoxetine. Homologues I and II constitute the SSRI moiety of the new molecules, the first one having a two-carbon basic chain and the second one having a three-carbon basic chain. Molecules I-IX constitute the moieties of known 5-HT$_{2A}$ receptor antagonism. Analysis of the biological evaluation of the synthesized molecules will provide valuable information about the structural features necessary to achieve a strong and highly selective binding to both target sites.

## Acknowledgements

Dr. Kevin G. Pinney would like to thank Baylor University for their support for this project though their Faculty Incentive Program, awarded from September 5, 2003 - May 31, 2004.

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# NOVEL SEROTONIN REUPTAKE INHIBITORS FOR AUTISM TREATMENT

## RESEARCH PLAN

### a. Specific Aims

The proposed research focuses on the design and synthesis a library of twenty or more novel bi-functional organic molecules, which combine 5-$HT_{2A}$ receptor antagonism and serotonin reuptake inhibition into one molecular entity. This will be done with the purpose of incorporating into one molecular entity the biological effects of selective serotonin reuptake inhibitors (SSRIs) and "atypical" antipsychotics, to create drug candidates that can provide synergism in terms of their potential efficacy over a wider variety of the core symptoms in patients with autism. The biological mechanism of action of SSRIs is known as serotonin reuptake inhibition while atypical antipsychotics, initially applied to the treatment of other psychiatric disorders such as schizophrenia, combine potent dopamine type 2 ($D_2$) and serotonin type 2 (5-$HT_{2A}$) receptor antagonisms. The research agenda will be accomplished by the following strategy:

1. The design and synthesis of hybrid (bi-functional) molecules by covalently coupling two basic fluoxetine homologues with different functionalized piperazines and piperidines using synthetic organic procedures. It is important to emphasize the fact that the two fluoxetine homologues constitute the structural moieties within the target molecules that will exhibit inhibition of serotonin reuptake activity, while piperazinyl or piperidinyl cyclic amines constitute the moieties of known 5-$HT_{2A}$ receptor antagonism. These two desirable activities for the treatment of autism will be combined into one molecular entity (see Schemes I-III).

2. The biological evaluation of the synthesized molecules, in collaboration with Nicholas V. Cozzi, Ph.D., will consist of three main assays:
   a. Neurotransmitter uptake assays for the human serotonin transporter
   b. Receptor binding assays for the serotonin 5-$HT_{2A}$
   c. Evaluation of agonist/antagonist activity to serotonin 5-$HT_{2A}$ receptor

3. In order to establish whether the new molecules represent valuable "lead" compounds, an analysis of their serotonin reuptake activity and their binding affinity towards the serotonin 5-$HT_{2A}$ receptor will be compared to those presented by currently used medications, such as fluoxetine and risperidone, respectively. If the two activities presented by the new molecules are similar or greater than those of the currently available medications, then the new molecules will be considered as potential leads for further *in vivo* investigations.

4. The construction of a structure-activity relationship database, through critical analysis of biological data obtained from the new drug candidates prepared through chemical synthesis, is of critical importance for reaching the goal of ultimately discovering new, critically relevant drugs for autism. This will provide valuable information about the structural features necessary to achieve a strong and highly selective binding to both target sites and will greatly contribute to further investigation of this kind of target drugs. It is important to emphasize that this is the first time, to the best of our knowledge, that the bi-functional molecule approach has been employed towards the treatment of autism. Therefore, this is an innovative approach to the pharmacological treatment of autism that has the strong potential of overcoming the drawbacks of two separate pharmacological treatments, which include high costs and undesired side-effects.

## b. Background and Significance

Autism is defined as a "pervasive developmental disorder",[1] which involves three fundamental impairments:[2]

a) impairments in social relations
b) impairments in communication and use of language
c) impairments in restricted and recurrent compulsive patterns of behavior and activities

Moreover, the latter are considered to be "integral and core components" of autistic disorder.[1]

As noted in Table 1, autism is a disorder that seriously affects individuals and families worldwide, and the outcome for most of those suffering from it is not encouraging without the appropriate care. Therefore, there is a mandate to alleviate, in a timely and cost-efficient manner, the psychiatric and behavioral symptoms of the individuals with this disorder since these symptoms interfere significantly with their ability to participate in educational, social, work, and family systems.

Table 1. Epidemiology of Autism[2]

| | |
|---|---|
| Cases per births | 1-5 per 10,000 births |
| Predominance | 3-4 times more common in males than females. |
| Risk groups | 75-fold greater risk among siblings. |
| Outcome | 5-10% of autistic children lead independent lives as adults, 25% improve with age but still require supervision; up to 70% of autistic adults are institutionalized. |

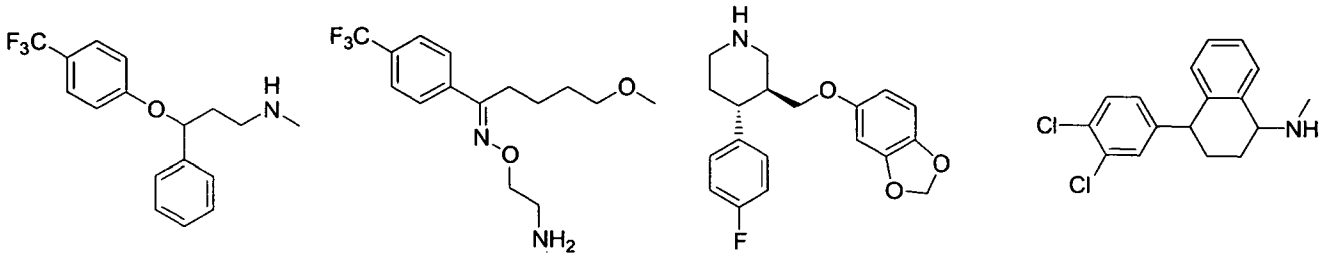
Persons with autistic disorder may present different clinical symptoms depending on their chronological age. Hyperactivity, stereotyped behaviors, irritability and temper tantrums may be the most prominent symptoms observed in patients in their early childhood, while ticlike behaviors, aggressiveness, and self-injurious behavior may develop later in the child's life. In adolescence and adulthood, depression and obsessive-compulsive phenomena are often observed.[3]

At the present time, there is general agreement between researchers that autism and related conditions are neurobiological disorders, although the specific biological cause has not yet been identified.[3,4,6] Therefore, no treatment specifically based on a cause has been developed in order to cure autism. Nevertheless, intensive research efforts have been made to establish the relationship between neurotransmitters and the clinical features of autism and other neuropsychiatric disorders, and preliminary findings strongly suggest that neurochemical factors play a major role in autism.[4]

Clinical evidence has shown that selective serotonin reuptake inhibitors (SSRIs) and "atypical" antipsychotics are drugs commonly used and proven effective in the treatment of some of the most important and incapacitating symptoms associated with autism spectrum disorders. SSRIs' mechanism of action is known as serotonin reuptake inhibition, and drugs belonging to this category such as fluoxetine, fluvoxamine, sertraline and paroxetine (Fig. 1) have been identified by researchers and patients' families as some of the most clinically useful agents,

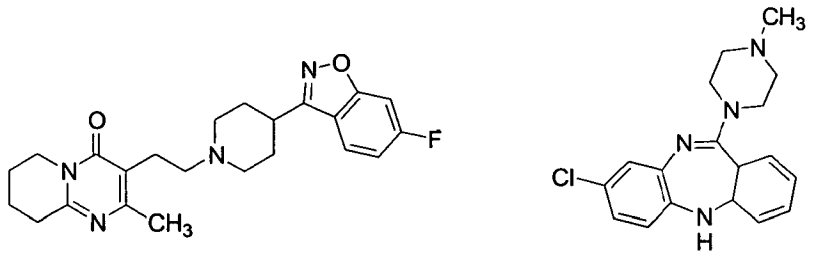
especially in targeting repetitive preoccupations, perseverative behaviors and anxiety-related symptoms.[5,6,7] At the same time, atypical antipsychotics such as risperidone and clozapine (Fig. 2) have also proven effective in improving other kinds of symptoms in patients with autism, such as hyperactivity, and in reducing the frequency and intensity of temper outbursts and aggression in patients with autism.[4] These drugs, initially applied to the treatment of other psychiatric disorders such as schizophrenia, are called "atypical" because they combine potent dopamine type 2 ($D_2$) and serotonin type 2 (5-$HT_{2A}$) receptor antagonism while "typical" or "first generation" antipsychotics only show dopamine type 2 ($D_2$) antagonism. Despite their relative effectiveness, all of the above currently available drugs still face an undesired side effect profile that limits their use, especially in the treatment of young children.[7]

**Figure 1.** Structure of selected SSRIs currently used in the treatment of autistic disorders.[1]




Fluoxetine Fluvoxamine Paroxetine Sertraline

**Figure 2.** Structure of selected atypical antipsychotics currently used in the treatment of autistic disorders.[4]

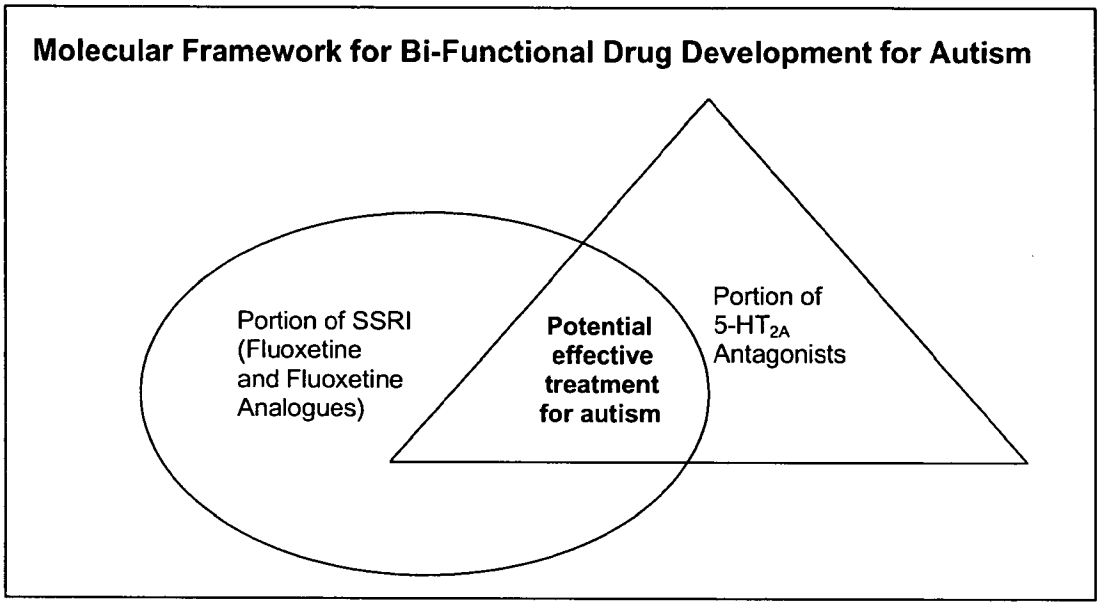



Risperidone Clozapine

Based on these clinical results, it is our hypothesis that by merging into one molecular entity serotonin transporter reuptake inhibition and 5-$HT_{2A}$ receptor antagonism activities, a superior pharmacological treatment for autism spectrum disorder may be achieved. A drug of this sort could not only combine the desired effects of two commonly used pharmacotherapies in the treatment of autistic symptomatology, but also show an improved side-effect profile and a lower maintenance cost.

In order to develop and evaluate molecules which can concomitantly antagonize the 5-$HT_{2A}$ receptors and block the serotonin transporter system, we have designed and plan to prepare a new series of molecules which judiciously combine portions of known 5-$HT_{2A}$ receptor antagonists with portions of SSRIs such as fluoxetine and related analogues. Our chemical strategy for constructing hybrid (bi-functional) molecules from 5-$HT_{2A}$ antagonists and serotonin reuptake inhibitors involves the "overlapping type" of approach,[8] as depicted in Figure 3.

**Figure 3.** Diagram showing the "overlapping type" approach for the design of bi-functional molecules.




With the data provided by this research, it is conceivable that significant advances will be made to the understanding of autistic syndrome. In addition, publications resulting from this study will aid in the timely communication of pertinent research results, and will promote further related studies, all aimed at finding new and effective treatments for the most important symptoms of autism.

## c. Preliminary Studies

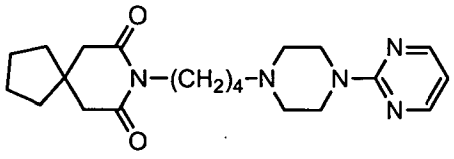
It is noteworthy that the Pinney Research Group has previous expertise in the design and synthesis of Serotonin-Selective Reuptake Inhibitors (SSRI) analogues. This work was published as a Master of Science thesis by James M. Dorsey, R.Ph. (*"Synthesis of Serotonin-Selective Reuptake Inhibitors With Novel Side Chains: Designing a Serotonin Reuptake Inhibitor with an Improved Adverse Reaction Profile"* Baylor University, 2001). *A recent publication of this work can be found at the Journal of Bioorganic and Medicinal Chemistry.*[9]

The project focused on the premise that SSRI-induced sexual dysfunction, a significant clinical problem associated with prolonged use of SSRIs, has the potential for successful treatment using pharmacological agents, and can ultimately be eliminated by the development of improved SSRI-type agents. More specifically, the strategy of coupling a structural portion of an SSRI with that of the pharmacophore of a pharmacological agent used in reversing sexual dysfunction may show promise in developing an improved SSRI-type agent.

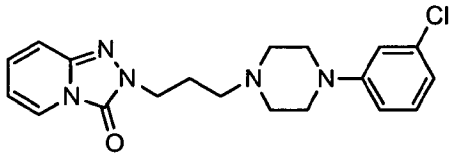
Sexual dysfunction is a common problem in affective disorders such as major depression. It is important to note, however, that the drugs used in the treatment of depression can cause sexual dysfunction, as well. Antidepressants such as the tricyclic antidepressant (TCAs), monoamine oxidase inhibitors, and SSRIs have all been reported to cause sexual side effects.[10] The inhibitory effects of the SSRIs on sexual function are well documented, and the frequency of occurrence varies widely. Some sources list ranges varying from 1% up to 75%[11] while the majority of sources estimate the occurrences in the 20-40% range.[12]

Strategies which have been tried in treating or reversing sexual dysfunction include: titration of an antidepressant dose, scheduling of a drug holiday during treatment, and administering a second drug (along with an SSRI, for example) to counteract the SSRI's sexual side effects.[12] The drugs which have been administered along with an SSRI include a broad range of medicinal agents. Drugs such as: buspirone (Buspar®), trazodone (Desyrel®), nefazodone (Serzone®), and sildenafil citrate (Viagra®) have been of benefit and their structures are depicted in Fig. 4.

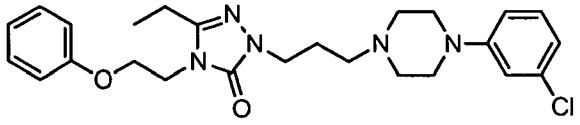
**Figure 4.** Structure of selected drugs administered with an SSRI in an effort to reverse/treat sexual dysfunction.



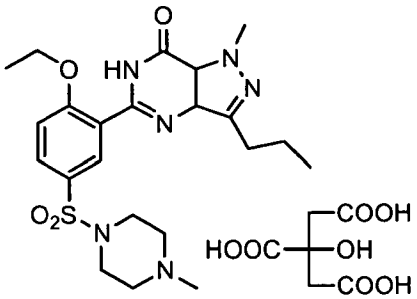

Buspirone




Trazodone



Nefazodone



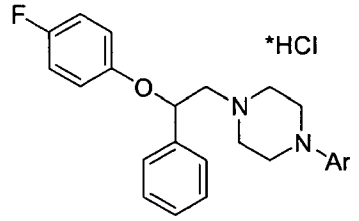
Sildenafil citrate

Buspirone, which has a pyrimidinyl-piperazine group, is generally used in the treatment of generalized anxiety disorder and has been used with some success in treating patients with SSRI-induced sexual dysfunction.[13] It is interesting to note that other piperazine-containing groups (specifically, functionalized phenylpiperazines) have likewise been used with success in treating sexual dysfunction. These agents include: sildenafil citrate (Viagra®), trazodone (Desyrel®), and nefazodone (Serzone®).

Based on the information that buspirone, trazodone, and nefazodone have all been used successfully in treating SSRI-induced sexual dysfunction, it was surmised that coupling the piperazinyl moiety to a structural homologue of an SSRI may have utility in synthesizing an SSRI candidate with a reaction profile devoid of sexual side effects. The strategy, therefore, was to covalently couple a structural homologue of the SSRI antidepressant, fluoxetine, with that of the piperazinyl-containing portion of each of the following compounds: buspirone, trazodone, and nefazodone. In the cases of trazodone and nefazodone, the piperazinyl-containing compound is a 1-(3-chlorophenyl)-piperazine. Buspirone, on the other hand, contains a 1-(2-pyrimidinyl)-piperazine moiety. Two other piperazine-containing candidates were investigated, as well. The first is m-(trifluoromethylphenyl)-piperazine and the other is 1-(2-methoxyphenyl)-piperazine.[9]

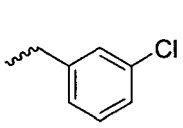
From this study, three novel SSRI-type molecules were designed, synthesized and evaluated for their serotonin reuptake activity (see Fig. 5).[9]

***Figure 5.*** *Structure and $IC_{50}$ values for serotonin uptake of the three 2-(4-fluorophenoxy)-2-phenyl-ethyl piperazines, novel SSRI-type molecules*

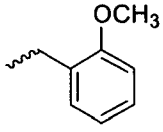


General structure for molecules **1**, **2**, and **3**

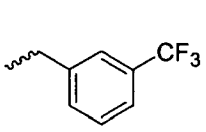
Where Ar:



for **1**
$IC_{50}$ = 1.45 μM



for **2**
$IC_{50}$ = 3.27 μM



for **3**
$IC_{50}$ = 9.56 μM

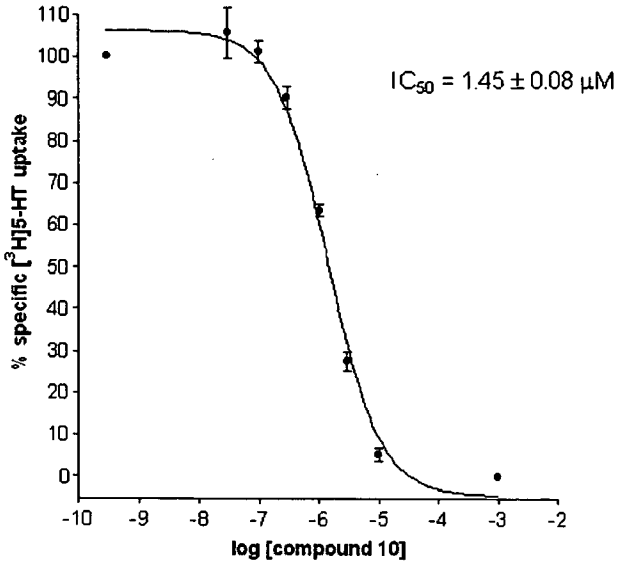



***Figure 6.*** *Graphical depiction of $IC_{50}$ data for compound **1** (by N.V. Cozzi) (Compound **1** was denoted as Compound 10 in recent publication)*

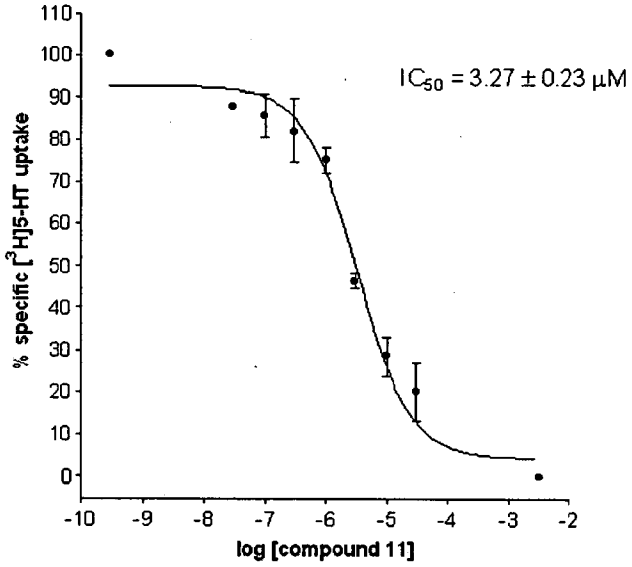



***Figure 7.*** *Graphical depiction of $IC_{50}$ data for compound **2** (by N.V. Cozzi) (Compound **2** was denoted as Compound 11 in recent publication)*

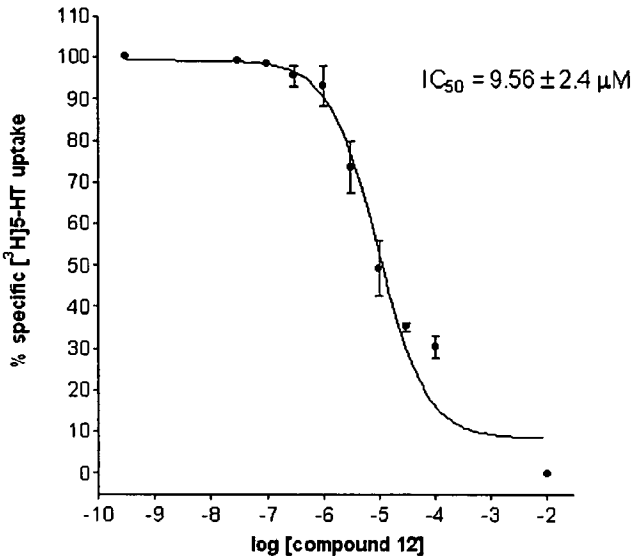



***Figure 8.*** *Graphical depiction of $IC_{50}$ data for compound **3** (by N.V. Cozzi) (Compound **3** was denoted as Compound 12 in recent publication)*

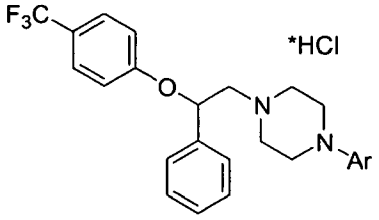
Although a complete biogenic amine activity profile with regard to the biological activity of these compounds remains to be elucidated, initial findings regarding the serotonin reuptake inhibition of the coupled piperazinyl-containing hybrids were determined. Preliminary data indicates that the hydrochloride (HCl) salts **1, 2,** and **3** each **exhibit single-site binding at the site of the serotonin reuptake transporter (SERT).** In contrast, each of the three compounds is **much less potent** than typical SSRIs such as citalopram, fluoxetine, or paroxetine which exhibit nanomolar (nM) affinity for the SERT[14] (see Fig. 6, 7 and 8).

Further biological evaluation of compounds **1, 2,** and **3** is needed before definitive conclusions can be made with regard to each compound's potential for use as an SSRI-type candidate which is devoid of sexual side effects. Nevertheless, the initial findings are quite encouraging, thus lending credence to the idea of hybridizing an SSRI congener with that of the pharmacophore of an agent known to reverse or treat SSRI-induced sexual dysfunction.

It is also important to note that biological evaluation of these three molecules was performed in collaboration with Nicholas V. Cozzi, Ph.D. (Department of Pharmacology and Toxicology, Brody School of Medicine at East Carolina University, Greenville, NC). Dr. Cozzi has already agreed to continue collaborating with the Pinney Group in its future endeavors, including the present proposal (Note: Letter of Collaboration is enclosed). This previous experience, along with the new and innovative ideas described herein, will provide the necessary background for the development of the proposed research project. A variety of target molecules has already been designed, which include hybrid molecules of arylpiperazine propiophenones with butyrophenone and benzisoxazole derivatives as side chains, among others.

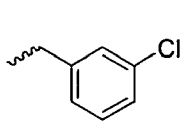
*In an attempt to further expand the previously described study, three novel 2-(4-trifluoromethylphenoxy)-2-phenyl-ethyl piperazines (Fig. 9) have been designed and successfully synthesized utilizing a new and more efficient synthetic method (See Synthesis of Homologues Type I, Research Design and Methods). These molecules, structurally similar to the previously described ones, retain the 4-trifluoromethylphenoxy moiety contained by the parent compound, fluoxetine (Fig. 1) while keeping a two carbon chain. Evidence has indicated in the past that para-substituens in the fluoxetine molecule, and particularly highly hydrofobic and electronegative ones like trifluoromethyl, are primarily responsible for the selectivity of fluoxetine for the serotonin transporter (SERT).[15] This structural modification was made with the purpose of establishing a structure-activity relationship (SAR) by examining if the potency of these molecules improves with it, and to what extent. The hydrochloride salts of the three compounds will be evaluated regarding their affinity towards the SERT and also, for screening purposes, regarding their affinity towards the 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors. The results of this biological evaluation as well as the details concerning the synthesis of our compounds will be published in a timely manner.*

***Figure 9.*** *Structure of three novel 2-(4-trifluoromethylphenoxy)-2-phenyl-ethyl piperazines.*

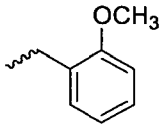



General structure for molecules **4**, **5**, and **6**

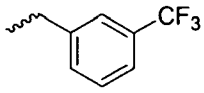
Where Ar:




**4**




**5**




**6**

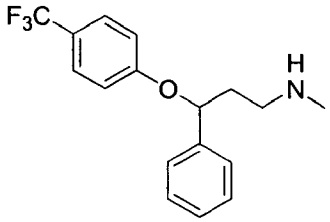
## d. Research Design and Methods

The proposed research focuses on the design and synthesis a library of twenty or more novel bi-functional organic molecules which combine 5-$HT_{2A}$ receptor antagonism and serotonin transporter reuptake inhibition into one molecular entity. Our chemical strategy for constructing these hybrid (bi-functional) molecules from 5-$HT_{2A}$ antagonists and serotonin reuptake inhibitors will be to covalently couple different functionalized piperazines and piperidines with two basic fluoxetine homologues using synthetic organic procedures.

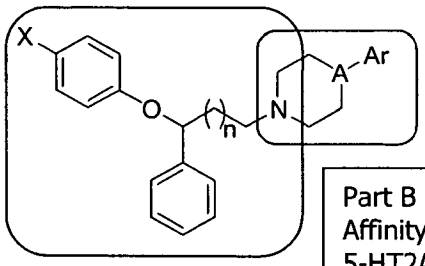
### 1) Design and Synthesis of Proposed Molecules

It is important to emphasize the fact that Homologues I and II (Schemes 1 and 2, respectively) constitute the structural moieties within the target molecules that will exhibit inhibition of serotonin reuptake activity, while cyclic amines I-V (piperazinyl or piperidinyl) constitute the moieties of known 5-$HT_{2A}$ receptor antagonism. These two desirable activities for the treatment of autism will be combined into one molecular entity.

**Rationale for Drug Design**




**Fluoxetine (Prozac®)**
Target: Inhibition of Sertonin Transporter

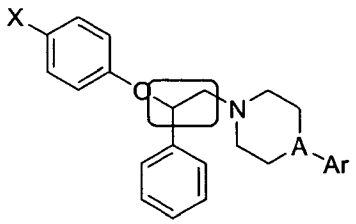
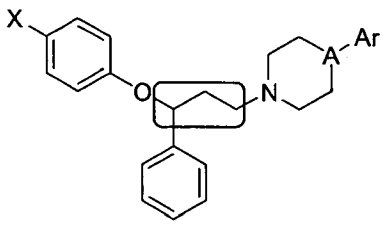



Part A → Affinity to SERT

Part B → Affinity to 5-HT2A receptors

**Novel Bi-functional SSRIs**
Targets:
a) Inhibition of Sertonin Transporter
b) Antagonism towards 5-HT2A receptors

X = F or CF3
N = 0 or 1 (ethyl or propyl chain)
A = N or C (piperazine or piperidine)
Ar = variety of aryl derivatives

| Homologues Type I | Homologues Type II |
|---|---|
|  |  |
| **Aminoethane Derivatives**<br>X = F or CF3<br>A = N or C (piperazine or piperidine)<br>Ar = variety of aryl groups | **Aminopropane Derivatives**<br>X = F or CF3<br>A = N or C (piperazine or piperidine)<br>Ar = variety of aryl groups |

Synthesis of Homologues Type I (see Scheme 1)

*Starting from commercially available 2-chloroacetophenone, covalent coupling of each cyclic amine will be carried out by a straightforward N-alkylation reaction in the presence of base. Reduction with sodium borohydride of the resulting ketones will provide the corresponding alcohols. Finally, Mitsunobu coupling of each alcohol with either 4-fluorophenol or 4-trifluoromethyl phenol will yield the proposed set of compounds.*

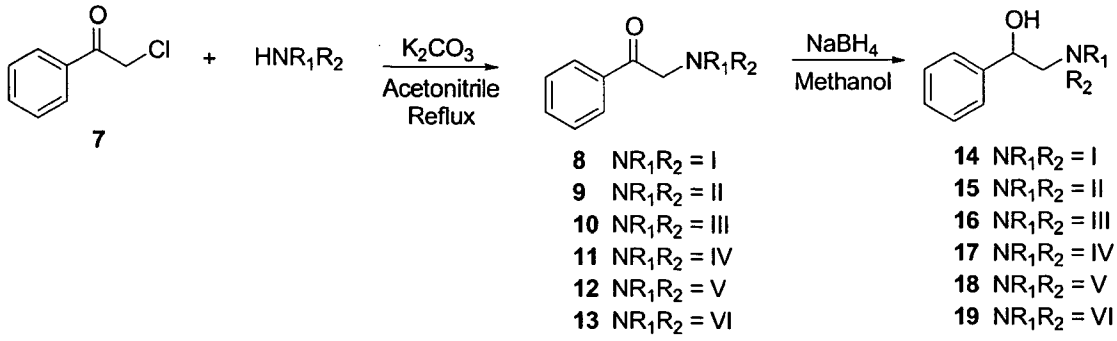
Synthesis of Homologues Type II (see Scheme 2)

Mannich reaction of acetophenone with the five proposed cyclic amines will give the corresponding 3-aminopropiophenones, which will be subjected to borohydride reduction to yield the corresponding aminopropanols.[16] *Mitsunobu coupling with either 4-fluorophenol or 4-trifluoromethyl phenol will yield the proposed set of compounds.*

Synthesis of Cyclic Amines (see Scheme 3)

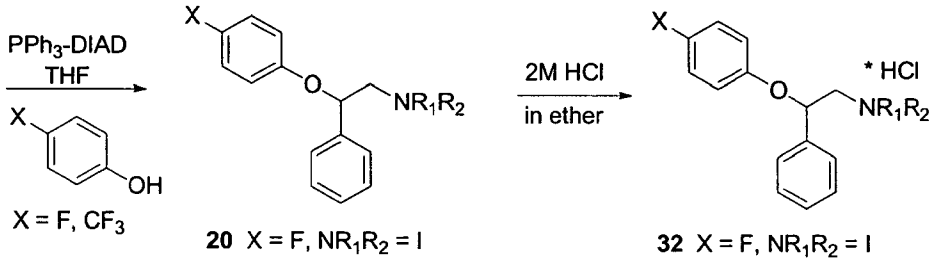
i. Amine I: Isonipecotoyl chloride will be reacted with 1,3-difluorobenzene under Friedel-Crafts conditions. From this product, the corresponding oxime will be generated, followed by cyclization with sodium hydride. Deprotection with acid will yield the desired final product.[19]
ii. Amine II: N-acetylisonipecotic acid will be treated with thionyl chloride, and the resulting acid chloride reacted with fluorobenzene under Friedel-Crafts conditions. The desired product will be achieved after acid hydrolysis of the acetyl protecting group.[18]
iii. Amine IV: N-alkylation of piperazine with p-chloro-butyrophenone under reflux will yield the desired product.[17]

***Scheme 1.*** *Synthesis of Homologues Type I*




**8** $NR_1R_2$ = I
**9** $NR_1R_2$ = II
**10** $NR_1R_2$ = III
**11** $NR_1R_2$ = IV
**12** $NR_1R_2$ = V
**13** $NR_1R_2$ = VI

**14** $NR_1R_2$ = I
**15** $NR_1R_2$ = II
**16** $NR_1R_2$ = III
**17** $NR_1R_2$ = IV
**18** $NR_1R_2$ = V
**19** $NR_1R_2$ = VI



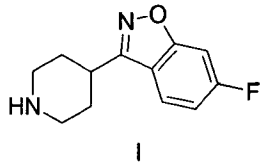

**20** X = F, $NR_1R_2$ = I
**21** X = F, $NR_1R_2$ = II
**22** X = F, $NR_1R_2$ = III
**23** X = F, $NR_1R_2$ = IV
**24** X = F, $NR_1R_2$ = V
**25** X = F, $NR_1R_2$ = VI

**26** X = $CF_3$, $NR_1R_2$ = I
**27** X = $CF_3$, $NR_1R_2$ = II
**28** X = $CF_3$, $NR_1R_2$ = III
**29** X = $CF_3$, $NR_1R_2$ = IV
**30** X = $CF_3$, $NR_1R_2$ = V
**31** X = $CF_3$, $NR_1R_2$ = VI

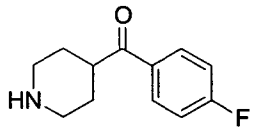
**32** X = F, $NR_1R_2$ = I
**33** X = F, $NR_1R_2$ = II
**34** X = F, $NR_1R_2$ = III
**35** X = F, $NR_1R_2$ = IV
**36** X = F, $NR_1R_2$ = V
**37** X = F, $NR_1R_2$ = VI

**38** X = $CF_3$, $NR_1R_2$ = I
**39** X = $CF_3$, $NR_1R_2$ = II
**40** X = $CF_3$, $NR_1R_2$ = III
**41** X = $CF_3$, $NR_1R_2$ = IV
**42** X = $CF_3$, $NR_1R_2$ = V
**43** X = $CF_3$, $NR_1R_2$ = VI

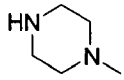
**Where $HNR_1R_2$ =**



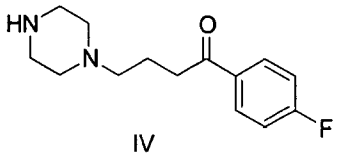

I




II




III

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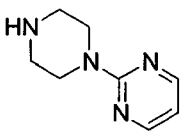
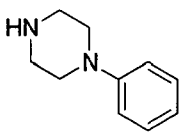
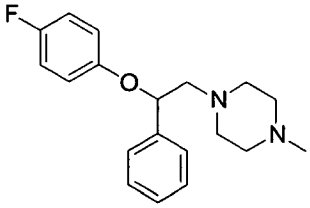



V

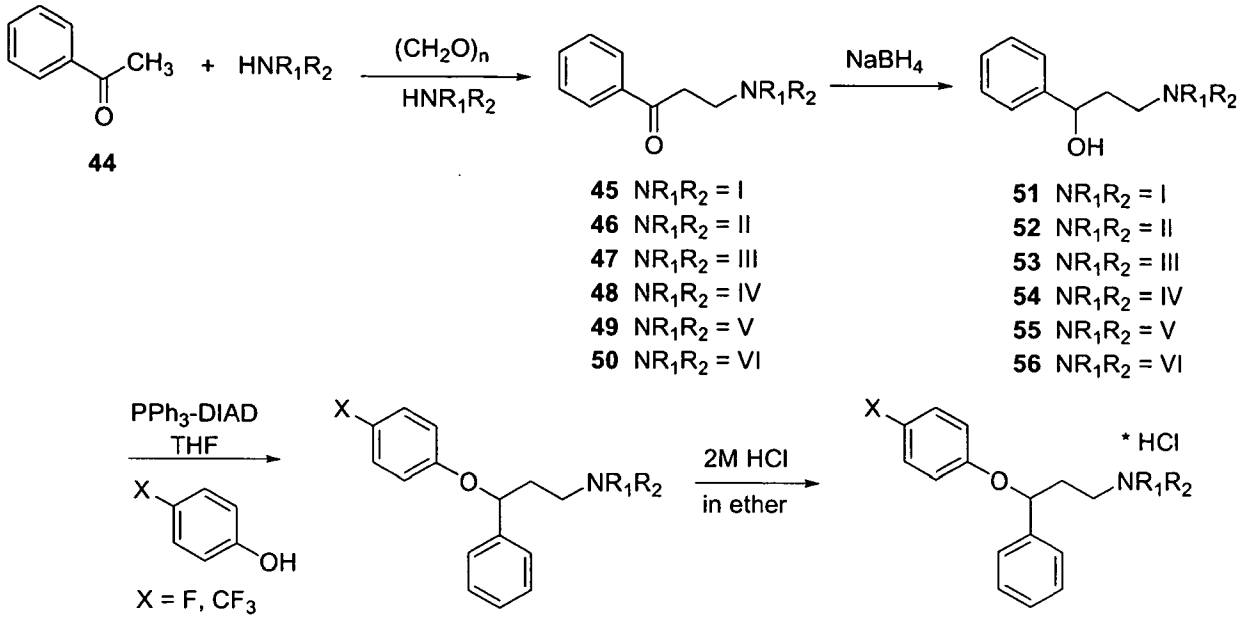



VI

**For example, Compound 22:**



***Scheme 2.*** *Synthesis of Homologues Type II*




**44**

**45** $NR_1R_2$ = I
**46** $NR_1R_2$ = II
**47** $NR_1R_2$ = III
**48** $NR_1R_2$ = IV
**49** $NR_1R_2$ = V
**50** $NR_1R_2$ = VI

**51** $NR_1R_2$ = I
**52** $NR_1R_2$ = II
**53** $NR_1R_2$ = III
**54** $NR_1R_2$ = IV
**55** $NR_1R_2$ = V
**56** $NR_1R_2$ = VI

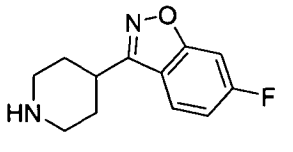
**57** X = F, $NR_1R_2$ = I
**58** X = F, $NR_1R_2$ = II
**59** X = F, $NR_1R_2$ = III
**60** X = F, $NR_1R_2$ = IV
**61** X = F, $NR_1R_2$ = V
**62** X = F, $NR_1R_2$ = VI

**63** X = $CF_3$, $NR_1R_2$ = I
**64** X = $CF_3$, $NR_1R_2$ = II
**65** X = $CF_3$, $NR_1R_2$ = III
**66** X = $CF_3$, $NR_1R_2$ = IV
**67** X = $CF_3$, $NR_1R_2$ = V
**68** X = $CF_3$, $NR_1R_2$ = VI

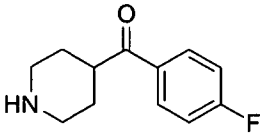
**69** X = F, $NR_1R_2$ = I
**70** X = F, $NR_1R_2$ = II
**71** X = F, $NR_1R_2$ = III
**72** X = F, $NR_1R_2$ = IV
**73** X = F, $NR_1R_2$ = V
**74** X = F, $NR_1R_2$ = VI

**75** X = $CF_3$, $NR_1R_2$ = I
**76** X = $CF_3$, $NR_1R_2$ = II
**77** X = $CF_3$, $NR_1R_2$ = III
**78** X = $CF_3$, $NR_1R_2$ = IV
**79** X = $CF_3$, $NR_1R_2$ = V
**80** X = $CF_3$, $NR_1R_2$ = VI

Where $HNR_1R_2$ =




I

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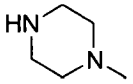
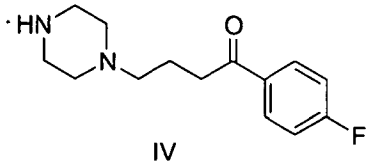
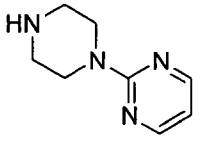


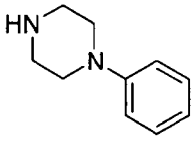

III




IV

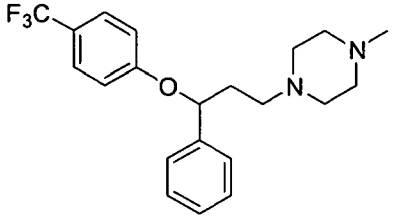



V



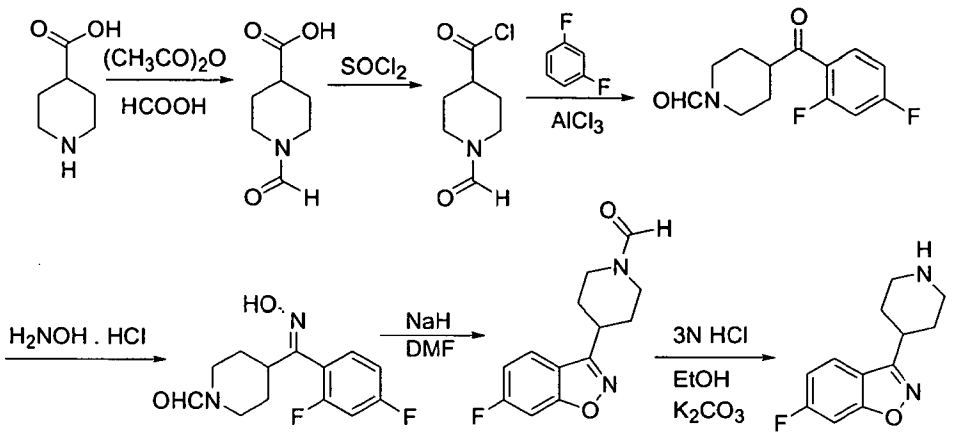

VI

**For example Compound 62:**

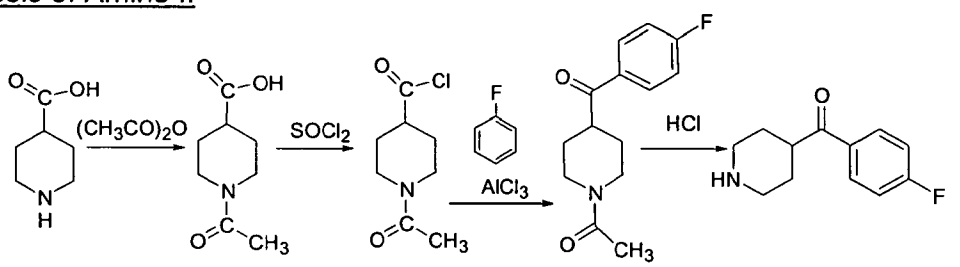


**Scheme 3.** Synthesis of Cyclic Amines I, II, and III

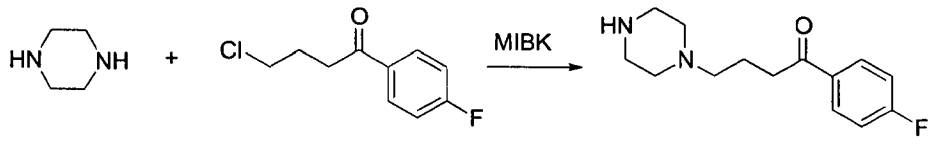
a) Synthesis of Amine I




b) Synthesis of Amine II




c) Synthesis of Amine IV




The identity and purity of the product of each step in the above syntheses will be obtained through [$^{1}$H] NMR, [$^{13}$C] NMR, [$^{19}$F] NMR, GC-Mass Spectrometry, elemental analysis and determination of melting point.

2) Biological Evaluation of Proposed Molecules

The biological evaluation of the synthesized molecules, in collaboration with Nicholas V. Cozzi, Ph.D., will consist of three main assays:

- Neurotransmitter uptake assays for the human serotonin transporter
- Receptor binding assays for the serotonin 5-$HT_{2A}$
- Evaluation of agonist/antagonist activity to serotonin 5-$HT_{2A}$ receptor

[$^3$H] 5-HT Uptake Procedure[9,20]

Outdated human platelets are obtained from a blood bank. Platelets from 5-10 donors are pooled, 10% dimethylsulfoxide is added, and aliquots are stored frozen at –80°C until use. For assays, 5 ml of platelets are thawed and added to 20 ml ice-cold Krebs-Ringer-HEPES (KRH) buffer containing (mM): NaCl (124.0), KCl (2.9), $MgSO_4$ (1.3), $KH_2PO_4$ (1.2), $CaCl_2$ (2.4), *d*-glucose (5.2), HEPES (25.0), sodium ascorbate (0.1), pargyline (0.1), pH = 7.4. The platelet suspension is subjected to centrifugation (4340 x g, 4 °C, 10 min) and the supernatant was discarded. The pellet is washed twice by resuspension in KRH and centrifugation. The final pellet is suspended in 20 ml ice-cold KRH containing 1% DMSO using a polytron (setting 4, 10 s) and stored on ice until use. The ability of platelets to accumulate [$^3$H]serotonin is measured in the absence and presence of test drugs as follows: a 490 µl aliquot of the platelet suspension is added to glass tubes containing 5 µl test drug (various concentrations, dissolved in DMSO), 5 µl KRH (for total determinations), or 5 µl fluoxetine hydrochloride (for nonspecific determinations; final concentration, 10 µM). The assay tubes are preincubated in a 37 °C shaking water bath for 5 min. The tubes are then returned to the ice bath and chilled for 10 min. [$^3$H]Serotonin was added (5 µl of stock solution; final concentration, 10 nM), giving a total incubation volume of 500 µl. All tubes are returned to the 37 °C shaking water bath for 5 min to initiate neurotransmitter uptake. Uptake is terminated by chilling the test tubes in the ice bath. After adding 3 ml ice-cold KRH, each assay tube is immediately vacuum filtered through glass fiber filters (Whatman GF/B) pretreated with 0.1% polyethyleneimine. Filters are washed with 2 x 3 ml ice-cold KRH, allowed to dry briefly under vacuum, and then placed in liquid scintillation vials. Scintillation cocktail (8 ml) is added and the vials were sealed, vortexed, and allowed to stand overnight. Radioactivity is measured using liquid scintillation spectroscopy (Packard Tri-Carb 1600 CA). Specific uptake is defined as uptake at 37 °C minus uptake in the presence of 10 µM fluoxetine. Under these conditions, specific [$^3$H]serotonin uptake is typically 90% of total uptake. The $IC_{50}$ value for each test drug is determined from displacement curves using at least 6 drug concentrations, each run in triplicate. Data are transformed from dpm to percentage specific uptake and fitted to a four-parameter logistic curve using commercial computer software, from which the $IC_{50}$ values are obtained.

*5-$HT_{2A}$ Binding Site Assay*[21,22]

*Three rat brains (minus cerebella, ~ 4 g wet weight) are used to prepare membranes by homogenization (Polytron setting 8, 2 x 20 sec) in 5 volumes ice-cold Krebs-Ringer-HEPES (KRH) buffer containing 100 uM pargyline and 100 uM ascorbate, pH = 7.4. The homogenates*

*are centrifuged at 1000 x g for 10 min at 4 oC. The pellet is discarded and the supernatant is centrifuged at 30,000 x g for 30 min at 4 oC. The resulting pellet is suspended in 5 mL KRH and aliquots are frozen at -80 oC until use. For binding assays, membrane aliquots are thawed and diluted with KRH to a final protein concentration of 30-50 ug/500 uL incubation volume. 6-9 concentrations of [125I]DOI (0.125-5 nM final concentration) are used for saturation binding assays. Membranes suspensions are added to test tubes containing KRH and various concentrations of [125I]DOI in the absence or presence of 10 uM cinanserin (to define nonspecific binding). Incubations are allowed to stand for 1 h at 0 oC, then the assay tubes are vacuum filtered through glass fiber filters pretreated with 0.1% polyethyleneimine. Filters are washed with 3 x 2 mL ice-cold KRH, then dried briefly under vacuum. The filters are placed into vials and radioactivity is counted in a gamma counter. Specific dpm are analyzed by nonlinear regression using commercially available computer software (GraphPad Prism) to determine Kd and Bmax.*

*Antagonism to Serotonin 5-$HT_{2A}$ Receptor*[21,22]

*For functional assays, the one to use for 5-$HT_{2A}$ activation is phosphoinositide hydrolysis and release of [3H]inositol phosphates. Cells or tissues are preincubated with 1 uCi/mL [3H]myo-inositol for 18 hours, washed, then treated with 100 uM pargyline and 10 mM LiCl for 15 min. After washing, agonists are added, incubated at 37 oC for 30 min, then the medium is discarded and the cells/tissues are solubilized in 10 mM formic acid. The [3H] inositol phosphates are separated from other material on a Dowex-1 ion exchange column and eluted into scintillation vials with 1 M ammonium formate and 0.1 M formic acid. Scintillation fluid is added and the vials are sealed, vortexed, and assayed for tritium in a scintillation counter.*

3) Criteria for Novel "Lead" Compounds

In order to establish whether the new molecules represent valuable "lead" compounds, an analysis of their serotonin reuptake activity and their binding affinity towards the serotonin 5-$HT_{2A}$ receptor will be compared to those presented by currently used medications, such as fluoxetine and risperidone, respectively. Both activities ($IC_{50}$ values) should be in the nanomolar range to be considered a "hit". If the two activities presented by the new molecules are similar or greater than those of the currently available medications, then those new molecules will be considered as potential leads for further *in vitro* and *in vivo* investigations. If the above activities are not in the nanomolar range (perhaps in the micromolar range), then those drug candidates will be subject to further chemical derivation in order to achieve a greater activity. These chemical derivations will be performed using the information from the structure-activity database (see next section). This database will provide valuable information about structural features necessary to achieve a strong and highly selective binding to both target sites.

4) Structure-Activity Relationship database

A structure-activity relationship database will be constructed through analysis of biological results of the new molecules. $IC_{50}$ values of serotonin reuptake activity and binding affinity towards the serotonin 5-$HT_{2A}$ receptor of the new molecules will be compared among themselves and also compared to those presented by currently used medications, such as fluoxetine and risperidone, respectively. This comparison will allow the researchers to establish the structural features necessary to achieve a strong and highly selective binding to both target sites, and will aid in further chemical derivation of those molecules that do not present potent enough activities in the first round of analysis. This database will be used as a guide for further target molecule design and selection, and will greatly contribute to further investigation of this kind of target drugs.

Tentative Timetable for the Proposed Research Project

| Start Date | End Date | Description |
|---|---|---|
| December 1, 2004 | June 30, 2005 | Synthesis of the first key proposed molecular targets (Dept. of Chemistry and Biochemistry at Baylor University) |
| July 1, 2005 | December 31, 2005 | - Continuation of synthesis of proposed molecular targets (Dept. of Chemistry and Biochemistry at Baylor University)<br>- Biological evaluation of the synthesized molecules (Dept. of Pharmacology and Toxicology at East Carolina University)<br>- Analysis of biological results in order to build the requisite structure-activity relationship (SAR) knowledge base regarding the key proposed molecules. This information will also be used as a guide for further target molecule design and selection (Dept. of Chemistry and Biochemistry at Baylor University)<br>- Preparation of First Year Progress Report |
| January 1, 2006 | November 30, 2006 | - Simultaneous design, synthesis, and biological evaluation of additional target molecules, and dissemination of the knowledge gained through this study by research presentations and timely publications<br>- Preparation of Final Progress Report |

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